Identification of Caffeic Acid in Cigarette Smoke

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No previous report has been made of the presence of caffeic acid (3, 4-dihydroxycinnamic acid) in cigarette smoke. Several groups of workers[1,2,3],

(1) F. Wilkinson, M. Phillips, and A. M. Bacot, J. Assn. Off. Agric. Chemists, 37, 1004 (1954).

(2) M. Shiroya, T. Shiroya, and S. Hattori, Physiol. Plant, 8, 594 (1955).

(3) M. K. Mikhailov, Compt. Rend. Acad. Bulgare des Sci., 11, 205 (1958).

however, have reported finding free caffeic acid in various cured tobaccos, but Roberts and Wood,[4] using fresh cigar tobacco, and Weaving,[5] using flue-cured

(4) E. A. H. Roberts and D. J. Wood, Arch. Biochem., 33, 299 (1951).

(5) A. S. Weaving, Tob. Sci., 2, 1 (1958).

tobaccos, could find none in their samples. Dieterman et al.[6] have recently

(6) L. J. Dieterman, C. H. Yang, Y. Nakagawa, and S. H. Wender, J. Org. Chem., 24, ___ (1959).

pointed out that esculetin (6, 7-dihydroxycoumarin) in tobacco may often be confused on paper chromatograms with caffeic acid. In the present study on tobacco in 8 brands of cigarettes commonly smoked in the U.S., every sample tested was found to contain free caffeic acid. Also, in every case, the mainstream smoke from the cigarette contained free caffeic acid.

In the purification of scopoletin (6-methoxy, 7-hydroxycoumarin) from cigarette smoke and from various tobacco extracts[7,8], two or more interfering

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(7) C. H. Yang, Y. Nakagawa, and S. H. Wender, J. Org. Chem., 23, 204 (1958).

(8) C. H. Yang, Y. Nakagawa, and S. H. Wender, Anal. Chem., 30, 2041 (1958).

blue fluorescing compounds persisted with the scopoletin through several developments of paper chromatograms. Dieterman et al.[6] identified one of these interfering compounds as esculetin. The present identification establishes free caffeic acid as the other blue fluorescing compound.

During paper chromatography in certain acid solvent systems, such as 15% acetic acid-water, caffeic acid appears as two distinct zones. These have been shown to be cis-and trans- caffeic acid.

EXPERIMENTAL

Caffeic acid from cigarette tobacco. The tobacco obtained from 120 cigarettes (3 each from 40 packs of the same brand) was mixed and ground to a powder. Six 5.5 g. samples of this powder were thoroughly extracted with 85% isopropyl alcohol, as previously described by Yang et al.[7] The combined extracts were concentrated under reduced pressure, and the concentrate was then subjected to separation by mass paper chromatography.[7] After the initial chromatography with Whatman 3MM paper, using the solvent system n-butyl alcohol-acetic acid-water (6:1:2 v/v), each zone containing caffeic acid, still mixed with some esculetin and scopoletin, was cut off and then eluted with methanol. The eluates were combined and streaked on S & S No. 589, Red Ribbon, chromatography paper, and developed in the system chloroform-acetic acid-water (2:1:1 v/v, bottom layer). This solvent system proved to be superior to the nitromethane-benzene-water system (2:3:5 v/v, upper layer) used in our previous studies on scopoletin and esculetin. In the chloroform system, the scopoletin (R_f=0.75) moves in a narrow zone quite removed from

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those of esculetin (R_f=0.39) and of caffeic acid (R_f=0.35). This was also the case with the benzene-propionic acid-water system (2:2:1 v/v, top layer) with R_f values: scopoletin (0.66); caffeic acid (0.32); and esculetin (0.26). The two top zones resulting from paper chromatography with the chloroform system contained primarily caffeic acid and esculetin. They were cut off from each chromatogram together; sewn onto a new sheet of paper; and then developed in ethyl acetate-formic acid-water (10:2:3 v/v). Each zone containing caffeic acid, with a trace of esculetin still present, was cut off and eluted with the ethyl acetate solvent system. The eluates were combined and again streaked on S & S No. 589 paper and developed in 15% acetic acid-water. Although separation of caffeic acid from esculetin was completed by this chromatography with acetic acid, an isomer of caffeic acid now appeared as a separate, third zone.

The two zones of isomeric caffeic acid were cut from each chromatogram as a unit and sewn onto a new sheet of chromatography paper. Each such sheet was then developed in the ethyl acetate system to obtain one narrow blue zone for identification studies.

Identification of caffeic acid. The combined eluates containing the purified caffeic acid from each single zone obtained in the ethyl acetate system were then co-chromatographed with authentic caffeic acid purchased from California Foundation for Biochemical Research, using the n-butyl alcohol-acetic acid-water, chloroform-acetic acid-water, ethyl acetate-formic acid-water, benzene-propionic acid-water, and nitromethane-benzene-water systems already described, and n-butyl alcohol-benzene-pyridine-water (5:1:3:3 v/v, upper layer), isopropyl alcohol-pyridine-acetic acid-water (8:8:1:2 v/v), and 15% acetic acid-water. Both the reference and isolated caffeic acid solutions gave the same R_f values in every test. In the 15% acetic acid system, both the reference and unknown caffeic acid samples gave two zones each, with corresponding R_f values.

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The isolated and reference caffeic acids behaved similarly towards the chromogenic agents previously reported.[6] In addition, both gave the same color reaction with the Höfner reagent[4] (pink, changing to yellowish-brown) and with 2% alcoholic ferric chloride solution (green, changing to gray).

The absorption spectra exhibited by the isolated caffeic acid in ethanol, and in buffer solutions at pH 3.5 and 6.8, checked in each case with the corresponding spectrum exhibited by the reference caffeic acid in ethanol and in buffer solutions at 3.5 and 6.8 in our laboratory and with those reported for these preparations by Sutherland.[9]

(9) G. K. Sutherland, Arch. Biochem. & Biophys., 75, 412 (1958).

Caffeic acid in the mainstream smoke from cigarettes. The smoking of 8 brands of cigarettes for caffeic acid analysis was performed by a procedure similar to the one already described for scopoletin in smoke by Yang et al.[8] Because caffeic acid, however, was present only in a trace amount in the smoke, samples representing smoke from 40 packs of cigarettes were combined and concentrated to obtain sufficient caffeic acid for unambiguous studies by paper chromatography. The separation, purification, and identification of caffeic acid from the cigarette smoke condensates were carried out by mass paper chromatography in the same manner as already described above for determination of caffeic acid in tobacco. Cigarettes analyzed included Camel, Lucky Strike, Philip Morris, Old Gold Straights, Pall Mall, Winston, Viceroy, and Oasis.

Isomerization of caffeic acid. On paper chromatography with 15% acetic acid-water, the reference caffeic acid gave two distinct zones. The farther moving zone (R_f=0.50) was called "CA-1", and the slower moving zone (R_f=0.42) was called "CA-2." Williams[10] has reported that cinnamic acid derivatives give two spots on

(10) A. H. Williams, Chem. & Ind., 120 (1955).

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paper chromatography with 2% acetic acid-water. He suggested that this was a case of cis-trans isomerization on paper. He did not, however, find out which spot corresponded to which isomer.

In our studies, we have streaked ethanol aliquots containing 100 µg. of the reference caffeic acid onto S & S No. 589 paper and developed these chromatograms in 15% acetic acid-water. Each blue fluorescing zone was cut out separately; sewn onto separate new sheets of paper; and again developed in the 15% acetic acid. It was observed that from the slower moving zone (CA-2), the faster moving zone (CA-1) was produced every time that the separated CA-2 was rechromatographed in this acid system. If this procedure, involving separation by paper chromatography, cutting, sewing, and rechromatography of the CA-2 was repeated five times, the original CA-2 zone from the 100 µg. sample of caffeic acid would change almost completely to CA-1, and the fluorescence of the CA-2 zone would practically disappear. On the other hand, the CA-1 zone produced only a relatively small amount of the CA-2 each time that the CA-1 was developed in the 15% acetic acid-water.

Both CA-1 and CA-2 co-chromatographed with the reference caffeic acid to give only one spot in all the solvent systems mentioned in this paper, except in the 15% acetic acid-water. In this latter system, the major spot from the reference caffeic acid on the first chromatograms was identical with CA-2, and the minor spot was the same as CA-1. Both CA-1 and CA-2 gave the same color reactions when tested with all the chromogenic agents described in this report.

Although the isomeric caffeic acids CA-1 and CA-2 could be readily obtained as completely separated zones on paper chromatograms, much difficulty was experienced in getting solutions of either isomer completely free of the other. During the preparation of such solutions by extraction or elution of the individual zones from the paper, and application of heat, isomerization was usually found to occur, and an equilibrium mixture was set up according to the temperature, solvent, etc.

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used. For the ultraviolet spectrophotometry, a solution consisting mainly of only isomer CA-1, and another preparation consisting mainly of only CA-2 were finally prepared, however, as described in the next paragraph.

Each isomeric zone was cut from the chromatogram separately and eluted with 95% ethanol in an elution chamber. Each eluate was then evaporated to dryness, in vacuo, without application of heat. The residue, containing the caffeic acid isomer plus a filter paper impurity, was then dissolved by 1 ml. of hot distilled water, added drop by drop, while the container was kept rotating. A blank solution containing the filter paper impurity, but no caffeic acid, was prepared in exactly the same manner as just described, except that no caffeic acid was present on the sheet of chromatography paper. The aqueous solution of each isomer was then added to cold distilled water in separate cuvettes, drop by drop, with a capillary tube. To the cuvette used as a blank, approximately an equal amount of the blank solution containing the filter paper impurity, but no caffeic acid, was added. The absorption spectra of these CA-1 and CA-2 solutions were then measured with the Beckman spectrophotometer, Model DU. Results are shown in Figure 1. The CA-1 preparation exhibited its high maximum at 278 mμ, whereas the CA-2 preparation had an even higher maximum at 340 mμ. Mixing of various amounts of CA-1 with CA-2 shifted the 340 mμ maximum of CA-2 to various corresponding lower wavelengths.

To interpret these results, one uses the working rule which states that when the absorption properties of the cis-trans isomers of a substance differ, "the more elongated isomer absorbs at somewhat longer wavelengths and more intensely".[11]

(11) A. E. Gillam and E. S. Stern, An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry, Edward Arnold Publishers Ltd., London, England 2nd. ed., 1957, p. 267.

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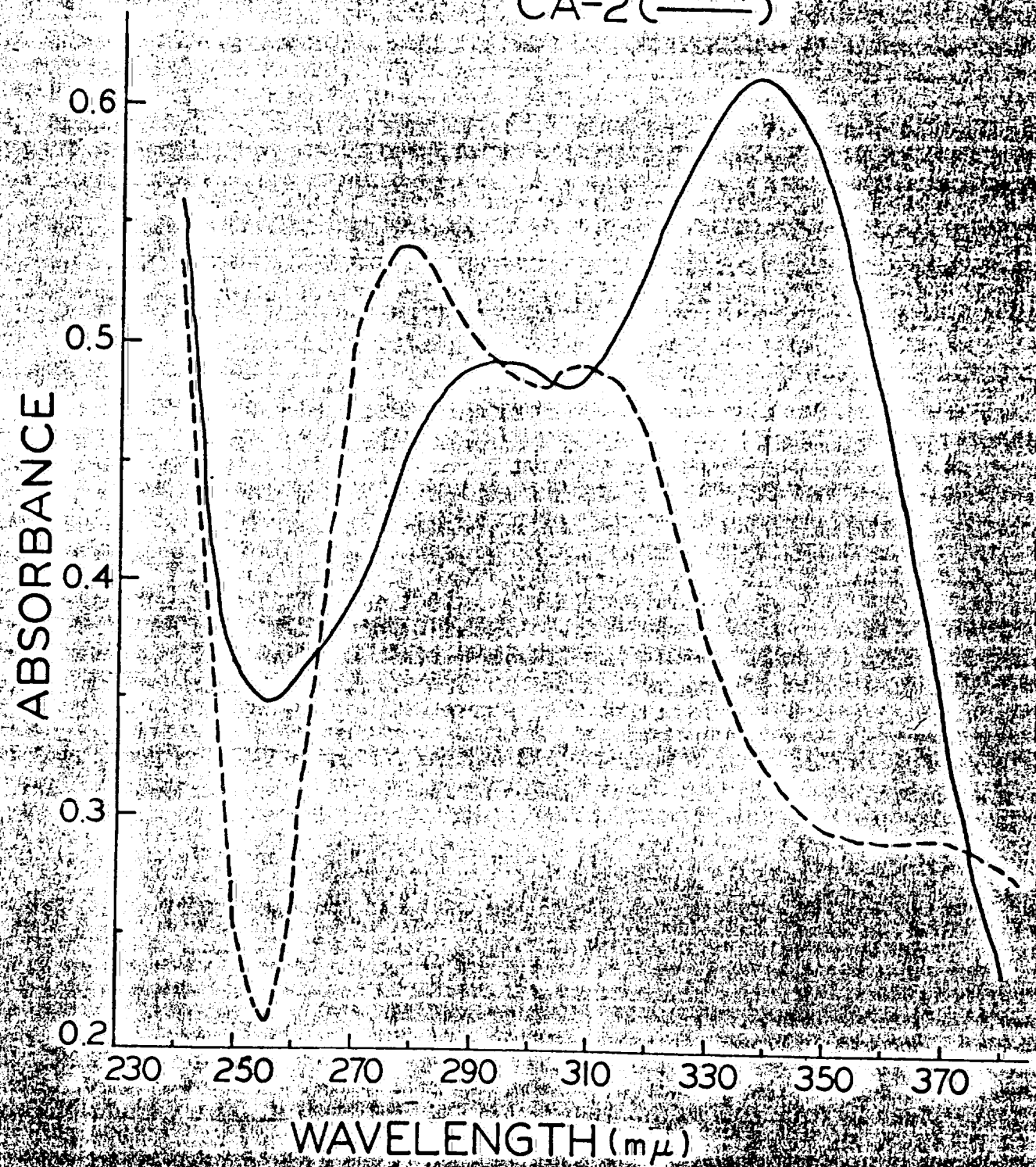
ABSORPTION SPECTRA OF AQUEOUS SOLUTIONS OF CAFFEIC ACID PREPARD FROM ZONES CA-1 (-----)
CA-2 (——)



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Haskins and Gorz[12] recently have found that such absorption data apply in their

(12) F. A. Haskins and H. J. Gorz, Arch. Biochem. & Biophys., 81, 204 (1959)

studies on cis- and trans - o-hydroxycinnamic acid. If this rule should also hold with caffeic acid, CA-1 would then appear to be the cis isomer and CA-2 the trans isomer of caffeic acid. Repeated paper chromatography in 15% acetic acid-water would, in such a case favor the isomerization of trans-caffeic acid to the cis form. The isopropyl alcohol extracts of cigarette tobacco and also the smoke condensates obtained in our research apparently already contain a mixture of the cis-trans isomers of caffeic acid even before paper chromatography with 15% acetic acid-water.

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